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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
We have developed a new adjuvant therapy for the control of metastatic breast cancer. This therapy exploits the involvement of cell-surface receptor bound urokinase plasminogen activator (uPA) in the metastatic spread of breast cancer cells. Once bound to specific cell-surface receptors, uPA efficiently activates plasminogen to the broad-spectrum protease, plasmin. We have successfully labeled and tested recombinant human PAI2 with the alpha radioisotope ^{213}Bi to produce alpha-PAI2. Low doses of alpha-PAI2 are highly cytotoxic towards breast cancer cell lines in vitro, whereas non-specific alpha-BSA had no cytotoxic effect. In vivo toxicity studies in nude mice show that up to 6 mCi/kg of alpha-PAI2 (ip) is well tolerated; in vivo efficacy experiments demonstrate in mice that a local injection of alpha-PAI2 can completely inhibit the growth of tumour at 2 days post-cell inoculation. At this time, only cell clusters are present. Further, a single systemic (iv) administration of alpha-PAI2 at 2 days post-inoculation can also inhibit tumour growth in a dose dependent manner, with 3/5 tumours uncontrolled at 1.5 mCi/kg, 2/5 at 3 mCi/kg and 1/5 at 6 mCi/kg. Clear evidence of tumour growth inhibition is established at 5 mCi/kg. Thus alpha-PAI2 is successful in targeting and killing isolated cells and preangiogenic cell clusters. These results indicate the promising potential of alpha-PAI2 as a novel therapeutic agent for micrometastatic breast cancer.

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INTRODUCTION

The major failure in breast cancer management is the incomplete killing of malignant tumour cells that have spread throughout the body [1]. This is despite the many treatments available, such as surgery, radiation therapy, hormone therapy and chemotherapy. The American Cancer Society estimated 182,800 new cases of invasive breast cancer in the year 2000 among women in America, and 40,800 are expected to die from the disease [2]. Novel, more effective treatments that overcome this problem in breast cancer management are essential. Targeted therapy, first discussed over 100 years ago, is based on the idea that a drug will attack its target without damaging other tissue [3]. Targeted alpha therapy (TAT) uses an alpha emitting radionuclide as a lethal medicament via an effective targeting carrier to kill cancer cells [4]. We are investigating a novel TAT approach that exploits the involvement of cell-surface receptor bound urokinase plasminogen activator (uPA) in the metastatic spread of breast cancer cells.

Alpha emitting radionuclides emit alpha particles with energies up to an order of magnitude greater than most beta rays, yet their ranges are two orders of magnitude less as alpha particles have a linear energy transfer (LET) which is about 100 times greater [5]. This is manifested by a high relative biological effectiveness (RBE). As a result, a much greater fraction of the total energy is deposited in cells with alphas and very few nuclear hits are required to kill a cell. Consequently, 100-fold enhancement in radiation dose [6, 7] would be delivered to the nucleus of a cancer cell if an effective carrier were employed to take the alpha radionuclide into that cancer cell. Thus, only alpha radiation has the potential to kill the metastatic cancer cells at tolerable dose limits, whereas the low LET of betas make this a very difficult task within human dose tolerance limits.

Availability of the alpha emitting radionuclides has been the major problem in the past for their large-scale scientific and clinical application. Studies have been carried out using ^{149}Tb [5,21], ^{211}At and ^{212}Bi [8-11] with encouraging results. The stable and reliable ^{225}Ac generator of the alpha emitting nuclide ^{213}Bi has been produced, modified and used successfully [11-22], with several of these studies indicating a therapeutic potential of ^{213}Bi-labeled antibody constructs against cancer cells both in vitro and in vivo. Our group has modified methods of conjugating ^{213}Bi radionuclide to antibodies with the stable chelator cyclic diethylenetriaminepentacetic acid anhydride (cDTPAa) for use in the alpha therapy of melanoma [18], colorectal cancer [21], leukaemia [22] and prostate cancer [48] and breast cancer [49].

A large body of experimental and clinical evidence implicates over-expression of the urokinase plasminogen activator (uPA) system as a modulator of the aggressive behaviour of tumour cells and as a strong prognostic factor for predicting poor breast cancer patient outcome [23-25]. uPA converts plasminogen into the highly active protease plasmin [23]. Plasmin promotes tissue degradation and remodelling of the local extracellular environment by directly and indirectly (via activation of pro-metalloproteases) degrading extracellular matrix molecules [23-25]. uPA is synthesised and secreted as a pro-enzyme, whose activation is markedly accelerated upon binding with high affinity (0.1 – 1 nM) to specific cell surface uPA receptors (uPAR) [23,26]. Receptor density varies depending on cell type (10^3 - 10^6 sites/cell) [26].

The ability of PAI2 to inhibit tumour invasion and metastases in animal models has been demonstrated by several laboratories utilising uPA-overexpressing cancer cells. For example, local invasion of human sarcoma xenografts is limited by stable expression of PAI2 in the sarcoma cells [42], over-expression of PAI2 in human melanoma cells inhibits spontaneous metastasis in immuno-compromised mice [43], and pre-treatment of rat mammary cancer cells with recombinant human PAI2 or slow infusion of the inhibitor with osmotic pumps led to a significant decrease in lung metastasis post intravenous administration [44]. Since ^{125}I-PAI2 was shown to accumulate in

uPA-overexpressing colorectal cancer cell xenografts in mice [36], and PAI2 levels were shown to be negligible in invasive colorectal cancer tissues that contained high levels of uPA antigen [45], it is likely that exogenous PAI2 can target invasive tumours in a uPA-dependent manner. Moreover, since localisation studies indicate that quiescent, normal or benign tissues do not contain significant levels of uPA it is unlikely that uPA-targeted therapy will appreciably affect normal tissues.

PAI-1 conjugated to A-chain cholera toxin as the cytotoxic agent or modified PAI-1 conjugated to saporin has been used to target fibrosarcoma cells [46,47] with moderate cytotoxicity. However, PAI2 has several distinct advantages over PAI-1 for targeted cancer therapy. Firstly, PAI2 is 10,000 fold less active than PAI-1 towards tissue type plasminogen activator, the latter having a high affinity for fibrin, indicating that administered PAI2 would not adversely affect fibrinolysis and hemostasis [35]. PAI2 is very stable in vitro compared to PAI-1 and does not revert to a latent form in vitro or in vivo compared to PAI-1 [35]. Sustained exposures to PAI2 are unlikely to cause adverse health effects since the "abnormally" high PAI2 levels found during late pregnancy (usually blood levels of PAI2 are undetectable) are not associated with toxicity [35]. In addition, obstacles associated with targeted immunotherapy, such as large protein size and human anti-mouse antibody responses, both of which require significant antibody manipulation to overcome such problems, are not a concern with PAI2.

Immunohistochemistry mirrored the endogenous uPA and uPAR antigen expression differences seen in two breast cancer cell lines, ie MDA-MB-231 and MCF7, by flow cytometry. Notably, the staining patterns of both antigens for both cell lines were punctuate and heterogeneous [49]. Most of the MDA-MB-231 cells were highly positive for either antigen with less than 10% being weakly positive. In contrast, most of the MCF-7 cells were only weakly positive with less than 10% being moderately positive.

Cell surface-bound uPA is accessible to and inhibitable by exogenous PAI2 [35,36], and a number of studies have suggested the potential for PAI2 to inhibit cancer cell invasion and metastasis [35].

The pharmacokinetics and biodistribution of human recombinant ^{125}I-labelled PAI2 in both control mice and mice bearing human colon cancer (uPA-positive HCT116 cell line) xenografts has been established [36]. Briefly, ^{125}I-PAI2 localised in 0.5 cm^3 tumour xenografts quickly (after 1 min, peaking at 30 - 60 min at approx. 1.5% of total injected dose). Furthermore, repeat intravenous, sub-cutaneous, or intra-peritoneal injections of ^{125}I-PAI2 resulted in an accumulation of radioactivity without an accompanying increase in the major organs or in toxicity. In addition, tumour associated ^{125}I-PAI2 correlated with tumour mass. Such studies indicate that invasive and metastatic tumour cells, shown consistently to contain active uPA, would be accessible to and targeted by exogenously administered PAI2.

It is clear that uPA is a specific marker of malignancy and that PAI2 represents a useful targeting agent. We have previously reported the production and evaluation of the new alpha-nuclide emitting cytotoxic agent ^{213}Bi-labeled PAI2 (alpha-PAI2) [49]. The reactivity and specificity of alpha-PAI2 cytotoxicity was reported for two human breast cancer cell lines in vitro. We now demonstrate the efficacy of TAT with alpha-PAI2 in inhibiting the growth of tumours in vivo, within dose tolerance limits. These data clearly show that alpha-PAI2 has an important role as a potential new therapeutic modality for the control of micrometastases in breast cancer.

MATERIALS AND METHODS

Materials

Human recombinant PAI2 (47 kDa) was provided by Biotech Australia Pty Ltd. Glu-plasminogen was purified from human plasma as described by Andronicos et al [37]. Microspin concentrators were purchased from Millipore (Bedford, MA, USA). RPMI-1640 was purchased from Life Technologies (Castle Hill, NSW, Australia). Fetal calf serum (FCS) was obtained from Trace Bioscientific (Castle Hill, NSW, Australia). The cyclic anhydride of diethylenetriaminepentacetic acid (cDTPA) was purchased from Aldrich Chemical Company. Bovine serum albumin (fraction V) (BSA) and propidium iodide (PI) were purchased from Sigma Chemical (St Louis, MO. USA). Human twin chain urokinase plasminogen activator (tc-uPA) was purchased from Serono (Sydney, NSW, Australia). Glu-gly-arg chloromethylketone (EGR-CMK) was purchased from Calbiochem (Sydney, NSW, Australia). Spectrozyme-UK (carobenzoxy-L-γ-glutamyl-(α-t-butoxy)-glycyl-arginine-p-nitroanilide-diacetate), mouse anti-human uPA IgG_1 (#394), mouse anti-human uPAR IgG_{2a} (#3696), and mouse anti-human PAI2 IgG_1 (#3750) monoclonal antibodies were purchased from American Diagnostica Inc (Greenwich, CT, USA). Mouse isotype control subclasses IgG_1, IgG_{2a} antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG were from Silenus (Sydney, NSW, Australia). Fresh human leukocytes were isolated from whole blood using Fycoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions.

Radioisotope

Alpha particle emitting radionuclide, ^{213}Bi, was eluted from the $^{225}Ac/^{213}Bi$ generator which was purchased from the Oak Ridge National Laboratory, United States Department of Energy. ^{213}Bi was eluted from the ^{225}Ac column with 250 µL of freshly prepared 0.15 M distilled and stabilized hydriodic acid followed by washing with 250 µL sterile distilled water [38]. The first elution was not used, and a time of 2 h was allowed for ^{213}Bi to regenerate on the column for the next elution. Corrections were made for ^{213}Bi decay using the half-life of 46 min for all activity calculations.

PAI2 conjugation with cDTPA, stoichiometry and reactivity

PAI2 and BSA were conjugated with cDTPA by a modification of the method described by Boll et al. [38] and Paik et al [39], to give the desired protein-DTTA conjugate. PAI2/BSA (1 mg) dissolved in PBS was conjugated to cDTPA by first increasing the pH to approximately 8.2 via the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9.0). A 50 fold molar excess of cDTPA (found to result in smallest fraction of underivatised PAI2; data not shown) was added and the mixture incubated at 25°C for 1 h with intermittent rocking. The reaction was stopped with a final concentration of 10% (v/v) 1 M Tris-HCl (pH7.2). The final reaction volume was 0.5 mL Three reaction volumes of PBS were used to purify DTTA-labeled proteins away from free cDTPA using a microspin concentrator as described by the manufacturer.

The concentrations of the protein-DTTA conjugates were measured by BIORAD DC protein assay reagent kit. The stoichiometry of DTTA-PAI2 was determined using electrospray ionisation mass spectrometry as previously described [49]. The DTTA-PAI2 was diluted in 1/20 in water and then ½ dilution with MeOH and 1% ascetic acid.

^{213}Bi labeling of DTTA-PAI2

Concentrated DTTA-PAI2 stocks were diluted with 500 mM sodium acetate at pH 5.5 and 5 – 10 µg of DTTA-PAI2 was labeled with free ^{213}Bi for 20 min at room temperature. After labeling, ^{213}Bi-DTTA-PAI2 (alpha-PAI2) was buffer exchanged into PBS using a PD-10 column using PBS (pH 7.0) as the eluting buffer. ^{213}Bi-DTTA-BSA (alpha-BSA) was radiolabeled by similar methods. The radiolabeling efficiency was determined by Instant Thin Layer Chromatography (ITLC), using a 10 µL aliquot of the final reaction mixture applied to Gelman paper (strip size 1 x 9cm, Gelman Science, Ann Arbor, MI). The paper strips were developed using

0.5 M sodium acetate (pH 5.5) as the solvent. The paper strips were cut into four sections and the gamma emissions from the radioisotope in each section was counted using a 340-540 keV window. The radiolabeled protein is found at the origin section, while free radioisotope is found at the solvent front section.

Stability of alpha-PAI2 was determined using ITLC to measure activity at the origin (labelled PAI2) and at the solvent front (free radioisotope) after incubation in serum and in DTPA challenge.

Cell culture

The metastatic MDA-MB-231 human breast cancer cell line was originally isolated from human breast adenocarcinona (pleural effusion) and does not carry characteristics of differentiated mammary epithelial [41]. Cells were routinely cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated FCS and passaged using Trypsin/EDTA. The cells were incubated in a humidified incubator at 37 °C with a 5% carbon dioxide air atmosphere. For all experimental procedures, sub-confluent cells that had been in culture for 48 h without a change of media were harvested by rinsing flasks twice with PBS (pH 7.2) and then detaching with PBS/0.5mM EDTA at 37 °C for 5 min. Cells were collected and resuspended in the appropriate buffer as described below.

Flow cytometry

For the detection of cell-surface uPA, uPAR and PAI2 indirect immunofluorescence staining was performed as described by Ranson et al. [41]. All flow cytometry data were analysed using CELLQuest software (Becton-Dickinson). Isotype control fluorescence is subtracted from all flow cytometry experiments.

Immunohistochemistry

The alkaline phosphatase anti-alkaline phosphate (APAAP) method [50] was used to detect uPA expression in MDA-231 cells after 2 days inoculation in nude mice. Briefly, paraffin-embedded tissues were cut at 5 μm sections, mounted on gelatin-coated glass slides (Davis Gelatine, Australia, Co) and then incubated for 20 min at 60°. The slides were deparaffinised in xylene, followed by a graded series of alcohols (100%, 95%, and 75%) and rehydrated in Tris-buffer saline (TBS, pH 7.5). The slides were immersed in preheated 10 mM citrate buffer (pH 6.0), boiled at high power in a microwave oven for 15 minutes and allowed to stand for 20 minutes at RT. The primary uPA (20 μg/mL) was incubated for 2 h at RT or overnight at 4°C. After washing with Tris buffered saline (TBS), slides including tissue sections were incubated with rabbit anti-mouse IgG (1:100 dilition) and APAAP complex (1:100 dilution) for 1 h and then stained by the addition of a fresh prepared alkaline phosphatase substrate included 0.2 mg/ml of naphthol AS-MX phosphate containing 0.1 mg/ml Fast Red TR and Levamisole in 0.1 M Tris HCl (pH 8.2) for 10 min. Control slides were treated in an identical manner. PC3 metastatic prostate cancer cells were chosen as a positive control while isotype MAb or the primary antibody omitted as a negative control. The positive cells appear pink.

Cytotoxicity assay

The CellTiter 96 Aqueous non-radioactive cell proliferation assay (Promega, WI, USA) was used to determine the effect of ^{213}Bi labeled proteins on cell survival.

The activities of alpha-PAI2 and alpha-BSA preparations were measured using a radioisotope calibrator and neutralized to pH 7.0 via the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9.0). Immediately after this, five serial doses of alpha-PAI2 and one dose of alpha-BSA were prepared in 100 μL RPMI/10%FCS and added to 96-well plates in triplicate containing 20,000 cells/well in 100 μL RPMI medium/10% FCS. The plates were then incubated overnight in a 5% carbon dioxide atmosphere at 37 °C. Controls were performed in triplicate in the same 96-well plate for each experiment and consisted of RPMI/10% FCS medium alone. In all cases the cells had been previously pre-incubated with 20 μg/mL plasminogen for 20 min at room temperature

and washed before placing into 96-well plates. In some cases, the cells were also treated with 0.5 mM EGR-CMK (a specific uPA inhibitor) [36] for 15 min at room temperature after plasminogen activation and prior to incubation with radiolabeled proteins.

The cells were then washed and incubated with 100 µL phenol-red free RPMI without FCS containing 20 µL of the CellTiter 96 reagent. After 3 h incubation in a 5% carbon dioxide atmosphere at 37 °C, the reaction was stopped by the addition of 10% SDS, and the absorbance in each well was recorded at 490 nm using a SPECTROmax plate reader. The absorbance reflects the number of surviving cells. Blanks were subtracted from all data and analysed using Prism software (GraphPad Software Inc, USA).

In vivo toxicity

Six weeks old Arc(s) nu/nu female mice were purchased from Animal Resources Centre, Western Australia. Groups of 5 mice received 1.5, 3 and 6 mCi/kg weight dose of alpha-PAI2 by i.p. injection, other mice were treated with PAI2, cDTPA, and saline as controls.

Biodistribution

Mice received an ip injection of alpha-PAI2 and were euthanised at 15, 30, 45, 60, 90 and 120 min. Tissues and organs were removed, weighed and the activity counted. The bone marrow count was obtained by measuring activity in the hip.

Two day model

One million cells were injected sc into the mammary glands of 5 mice, and at 2 days the mice were sacrificed and the local tissue section removed for histochemistry, as described above. The objective of this study was to demonstrate the state of pre-tumour development.

In vivo efficacy

Control injections were of PBS and non-specific ^{213}Bi-BSA and ^{213}Bi-monoclonal antibody.

Local TAT – dose response

Efficacy studies of local TAT were made for dose response at 2 days post-inoculation, and for post-inoculation time response. The alpha-PAI2 was injected in the same region as the inoculation, although no tumour was evident.

Local TAT – time response

Four different therapy time points were used, each with 5 mice: 2-4 days, 7 days, 14 days, and 28 days after cell inoculation. Each group had one control mouse and 4 treated mice.

Systemic TAT

The dose response was studied for systemic administration via ip injection at 2 days post-inoculation. Previous studies had shown little difference between ip and tail vein injections, so the more difficult tail vein approach was not justified.

Doses of 1.5, 3 and 6 mCi/kg were administered for dose response, and at 5 mCi/kg for efficacy. All in vivo studies were approved by the University of NSW Animal Care and Ethics Committee.

RESULTS

Chelation

Mass spectroscopy results are shown in Figure 1 for a) PAI2 alone, and b) DTTA-PAI2. Up to 5 fold attachment of cDTPA is observed, the peaks being separated by the MW of the chelator (357 D).

Stability of alpha-PAI2

ITLC results show that alpha-PAI2 is less stable in serum than in DTPA, and that most of the leaching occurs within one half-life of the radioisotope (Figure 2).

Cytotoxicity of alpha-PAI2 towards the MDA-MB-231 cell lines

Alpha-PAI2 was found to be highly toxic to MDA-MB-231 (Figure 3). In contrast, alpha-BSA showed only slight toxicity compared with alpha-PAI2 at the maximum activity used. No

significant toxicity was observed with either DTTA-PAI2 or PAI2. The D_0 (37% cell survival) values with alpha-PAI2 were calculated to be 2.1 ± 0.2 μCi (n = 6) for the MDA-MB-231 cell line.

Tolerance study

The weights of treated mice reduced initially by 5-10%, than recovered after 1 week. Mice were monitored and weight measured. After 13 weeks, the saline control mouse died, but other mice lived very well until euthanasia at 24 weeks post-therapy. Results for the 6mCi/kg group are shown in Figure 4.

Biodistribution

Results were obtained over two half lives (Figure 5) and showed that the kidneys received the highest activity, being more than half the observed activity after 25 min. The bone marrow receives the next highest dose, but other organs have relatively low exposure.

Two day in vivo model

One million MDA-MB-231 cells were injected subcutaneously into the mammary gland, and the tissue was resected at two days. Immunostaining with the #394 mab against uPA shows that isolated cells and cells clusters are prevalent, and there is no evidence for microcapillary formation (Fig 6). Thus the 2 day model accurately simulates micrometastasis and pre-angiogenic lesions.

Xenograft model

Xenografted tumours were induced successfully at 28/32 cell inoculation sites, the first tumours becoming visible at around 2-3 weeks post-inoculation. Tumour area of 1x1 cm^2 is reached after 4-6 weeks, at which time mice are euthanised. Histopathological analysis confirmed breast cancer cells in the tumours and metastatic cancer cells were found in the lymph nodes. Thus a human breast cancer model, including a lymphatic pathway metastasis model, was confirmed.

Local TAT: dose response

Local injection at 2 days of 12, 25 and 50 μCi of alpha-PAI2 showed a dose dependent response in groups of 5 mice. Control tumours (PBS) grew quickly, while the 12 μCi group grew very slowly. The 25 and 50 μCi groups showed complete inhibition of tumour growth (Figure 7).

In a second study, 50 μCi local alpha-PAI2 was injected into bilateral fat pads, using ^{213}Bi-BSA injection at 50 μCi as control. Complete inhibition of tumourogenesis was observed out to 34 days, but not for the controls (Fig 10).

Local TAT: post-inoculation times

A single injection of alpha-PAI2 (25 μCi) was made into cell inoculation sites or tumours. Mice were monitored and tumours were measured. The 2-4 day group had the best response to the therapy, which had around 50% (23/40) tumour control and slower tumour growth rate compared with control mice. 7 day and 14 day groups had 2/8 and 1/8 tumour control and slower growth rate compared with control mice. The 28 day group had 0/8 tumour control and no obvious change in tumour growth rate.

Systemic TAT

Mice received single ip injections of 25, 50 and 100 μCi alpha-PAI2 at 2 days post-inoculation. Results are shown in Fig 8 and indicate a substantial inhibition of tumour growth up to 50 days post-inoculation. The control represents a non-specific alpha-monoclonal antibody 9.2.27 against melanoma at 100 μCi, which has no effect on tumour growth. A similar result is observed for ^{213}Bi-DTTA-BSA, which has comparable mass to ^{213}Bi-DTTA-PAI2.

A dose effect is indicated in that increased activity decreased the number of tumours, ie 3/5, 2/5 and 1/5 for 25, 50 and 100 μCi activity (Figure 9).

A second study for a single 100 μCi ip injection (ie 5 mCi/kg) with ^{213}Bi-BSA and PBS as controls, showed that alpha-PAI2 induced a large growth delay in tumour growth up to 34 days (Fig. 10). Beyond this, point both control and alpha-PAI2 treated mice were sacrificed.

DISCUSSION

A number of novel techniques that target the uPA system for tumour therapy have been suggested and are being investigated [25]. We have investigated the use of PAI2 as the basis for a new therapeutic agent. In this study we describe for the first time the novel compound alpha-PAI2 and show that it retains reactivity and selectivity towards uPA expressing breast cancer cells in vitro.

That alpha-PAI2 cytotoxicity is significantly mediated via a uPA-dependent mechanism was further confirmed by the lack of cytotoxicity of freshly isolated normal human leukocytes on which cell-surface localised active uPA was not detectable [49]. Furthermore, breast cancer cells incubated with the non-specific alpha-BSA were also minimally affected. Clearly, alpha-PAI2 is very toxic to targeted cancer cells, whereas non-targeted cells are spared from the radio-toxicity arising from the alpha radiation. These results underscore the potential usefulness of alpha-PAI2 in vivo.

The in vivo studies revealed that alpha-PAI2 can target isolated cells and preangiogenic cell clusters, as is the situation at 2 days post-inoculation of breast cancer cells. Local therapy required only 25 μCi to achieve complete inhibition of tumour genesis. A much higher dose of 100 μCi was required for systemic administration to achieve 80% control. Alpha-PAI2 is increasingly less effective in a single dose protocol as the tumours grow in size. Clearly, the potential role of alpha-PAI2 lies in its ability to target and kill the most malignant cells that participate in the generation of micrometastases.

CONCLUSIONS

We have combined the cytotoxicity of an alpha-emitting radioisotope (^{213}Bi) with the targeting potential of PAI2 towards the uPA system to create a novel construct alpha-PAI2, a potential new therapeutic agent for targeted alpha therapy of cancer. The in vitro cytotoxicity of alpha-PAI2 on breast cancer cells was shown to be specific by several means indicating that the cell killing ability of alpha-PAI2 depends critically on the targeting of cells in a receptor bound, active uPA-dependent manner. The in vivo results show conclusively that alpha-PAI2 can target and kill isolated cells and cell clusters, and as such, is indicated for the control of micrometastic breast cancer.

FIGURE LEGENDS

Figure 1 Mass spectroscopy results for a) PAI2 and the b) chelated DTTA-PAI2. Multiple chelation occurs as is evident by the multiple peaks shifted by the MW of the chelator cDTPA of 357 D.

Figure 2 Stability of alpha-PAI2 in serum and in DTPA challenge. Percent loss of label is shown, as well as percent leaching with time. Most of the instability occurs within one half life of the conjugate.

Figure 3 Cytotoxicity study of MDA-MB-231 cells incubated overnight with varying concentrations of alpha-PAI2 (squares) or a single concentration of alpha-BSA (triangle), cell survival measured and expressed as a percentage of survival of control cells in RPMI medium alone. Values shown are the means of two experiments performed in triplicate.

Figure 4 Toxicity studies at 6 mCi/kg show no long term weight loss in nude mice after iv injection.

Figure 5 Biodistribution of alpha-PAI2 in nude mice. The kidneys receive the bulk of the activity.

Figure 6 Two day inoculation model sections showing immuno-staining of MDA breast cancer cells with a) monoclonal antibody #394 against uPA, compared with b) control stain with no primary antibody.

Figure 7 Inhibition of tumour growth by local injection of alpha-PAI2 at 2 days post-inoculation of $2x10^6$ MDA cells. An indicative dose response is apparent.

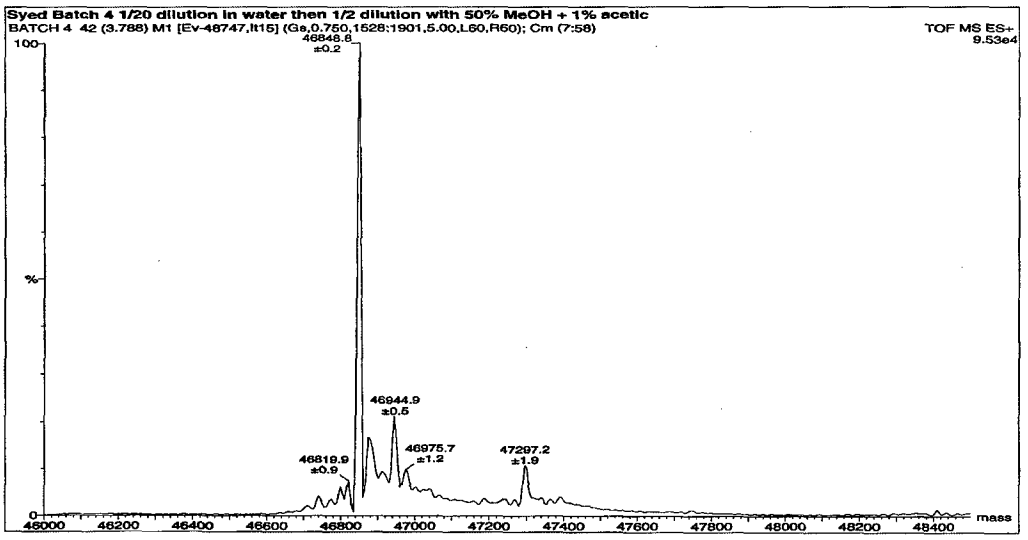
Figure 8 Alpha-PAI2 and non-specific alpha-monoclonal antibody injected at 2 days post-inoculation of human MDA breast cancer cells in nude mice (n=5 per group).

Figure 9 Effect on tumour growth after systemic (ip) administration of alpha-PAI2 at 2 days post-inoculation of MDA breast cancer cells, showing an indicative dose response, with 4/5 tumours being controlled at 6 mCi/kg.

Figure 10 Efficacy of alpha-PAI2 for local and systemic inhibition of pre-angiogenic breast cancer lesions in the 2 day inoculation nude mouse model. The control curve ( ) includes averaged results of tumour area after local and systemic injections of PBS and ^{213}Bi-BSA, which are not significantly different. In local TAT (Δ), bilateral injections of 50 μCi alpha-PAI2 are made, whereas for systemic TAT (∇), a single ip injection of 100 μCi is made.

Figure 1 Mass spectroscopy results for a) PAI2 and the b) chelated DTTA-PAI2. Multiple chelation occurs as is evident by the multiple peaks shifted by the MW of the chelator cDTPA of 357 D.

a)



b)

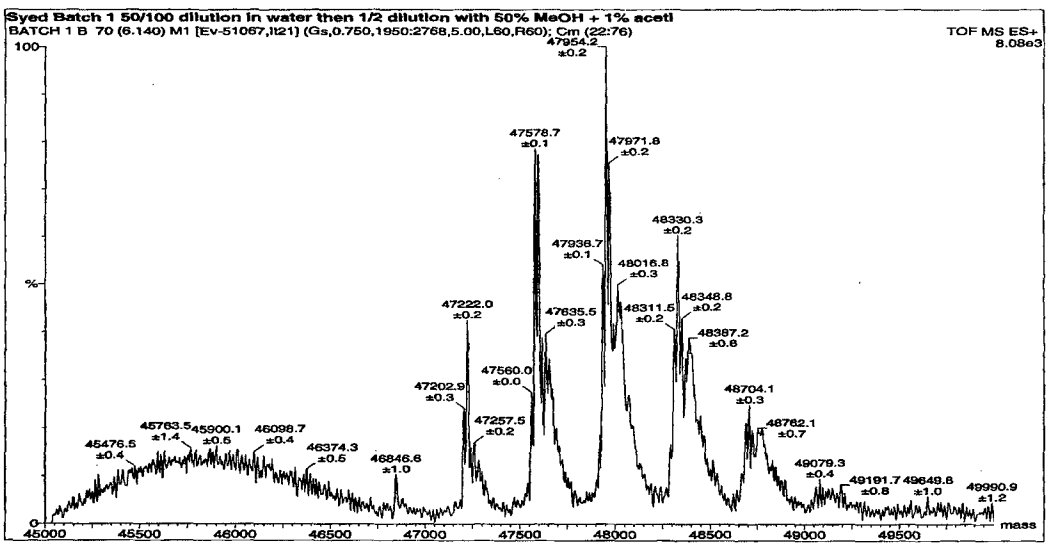


Figure 2 α-PAI2 stability test against DTPA challenge and serum

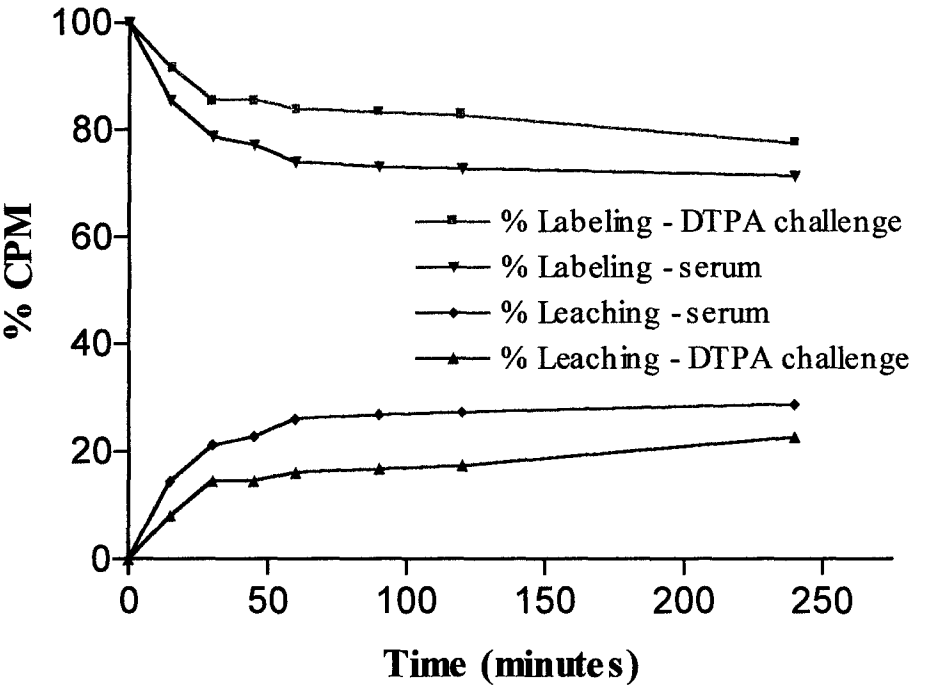


Figure 3 Cytotoxicity study of MDA-MB-231 cells incubated overnight with varying concentrations of alpha-PAI2 (squares) or a single concentration of alpha-BSA (triangle), cell survival measured and expressed as a percentage of survival of control cells in RPMI medium alone. Values shown are the means of two experiments performed in triplicate.

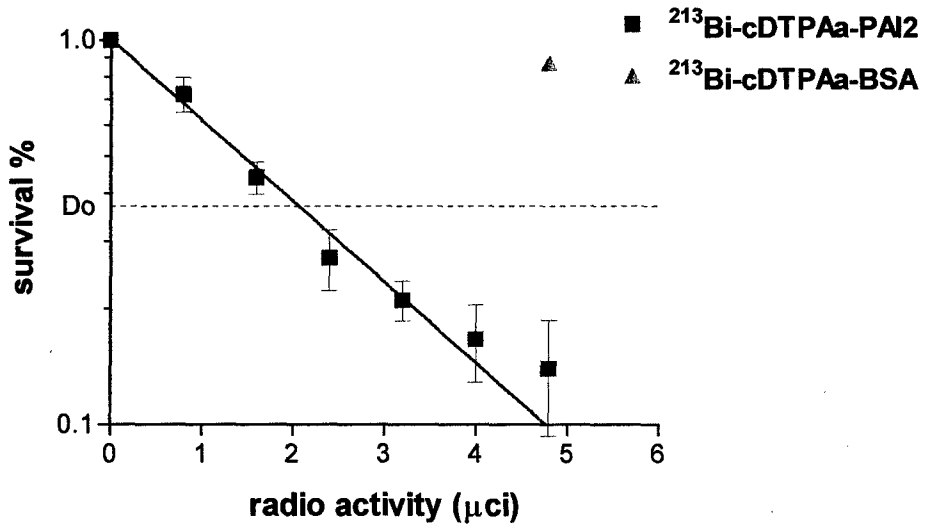


Figure 4. Toxicity studies at 6 mCi/kg show no long term weight loss in nude mice after iv injection.

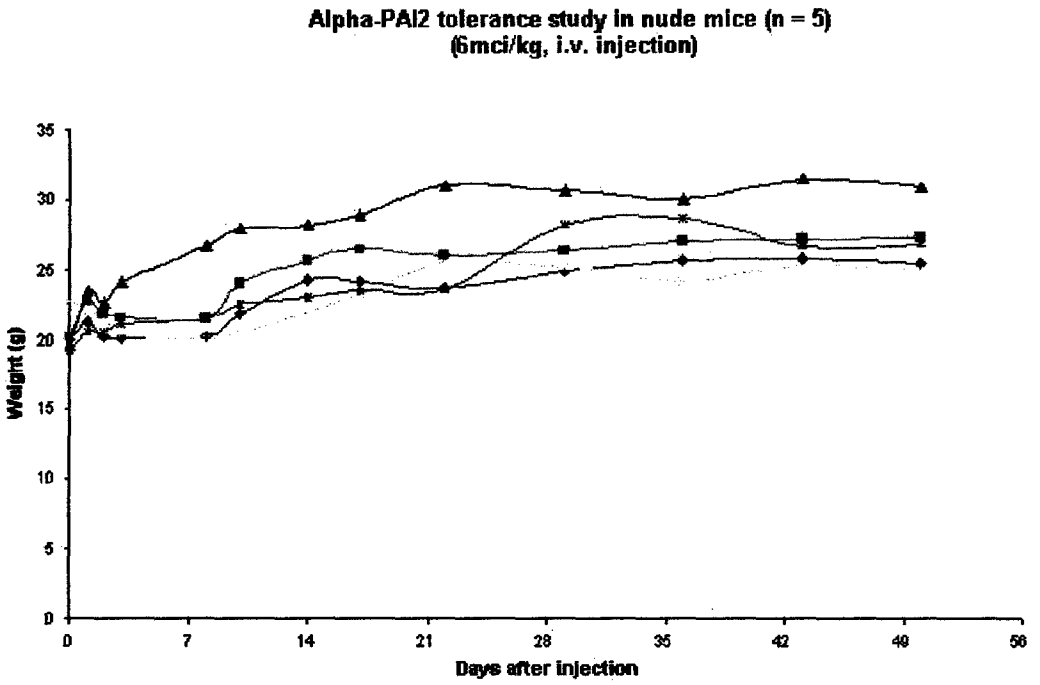


Figure 5 Biodistribution of α-PAI2 in nude mice

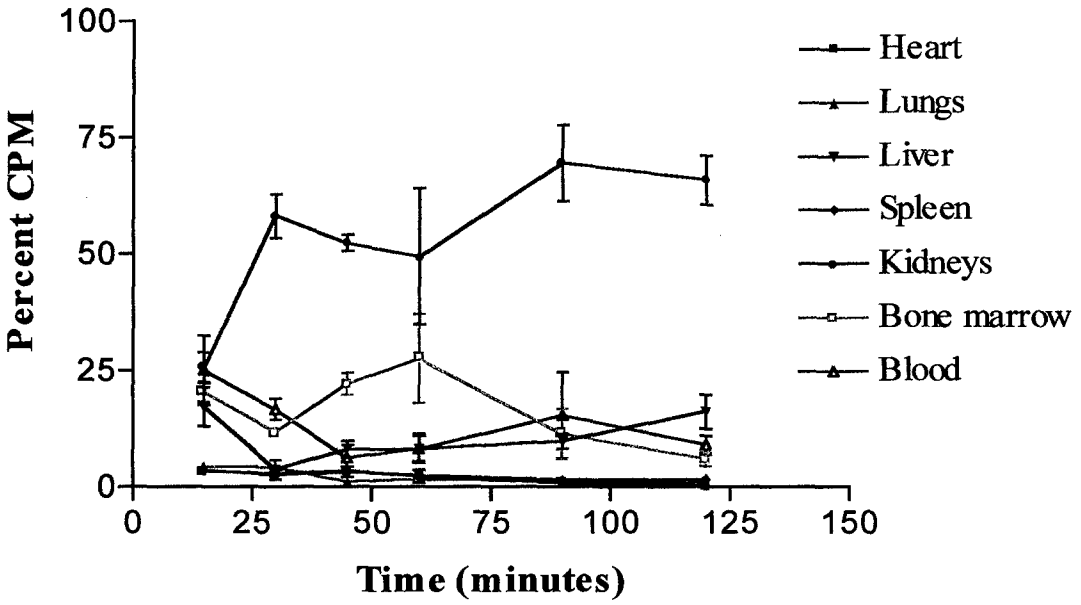
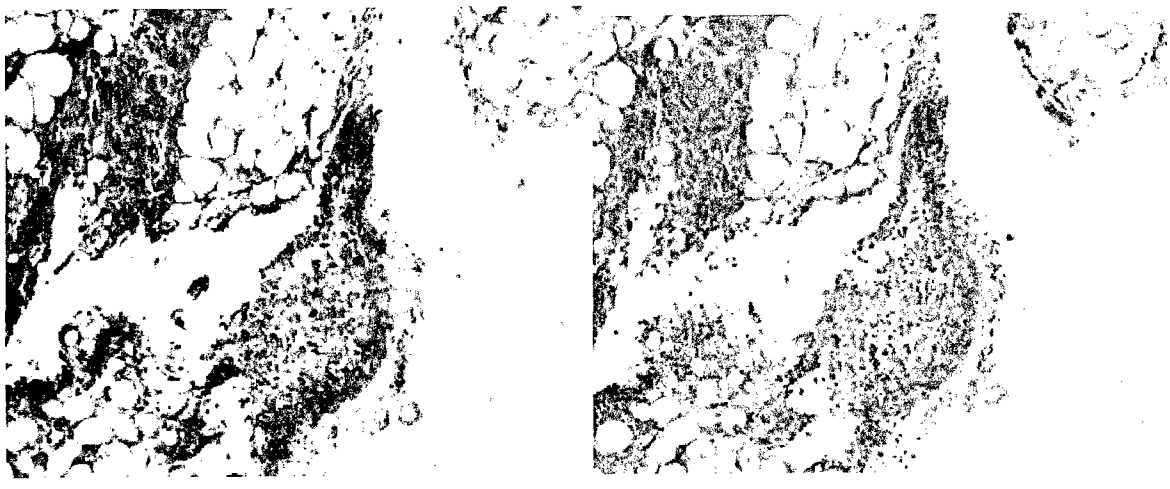


Figure 6 Two day inoculation model sections showing immuno-staining of MDA breast cancer cells with a) #394 mab against uPA, compared with b) control stain with no primary antibody.



A uPA #394 mab

B uPA control (no primary antibody)

Figure 7 Inhibition of tumour growth by local injection of alpha-PAI2 at 2 days post-inoculation of $2x10^6$ MDA cells. An indicative dose response is apparent.

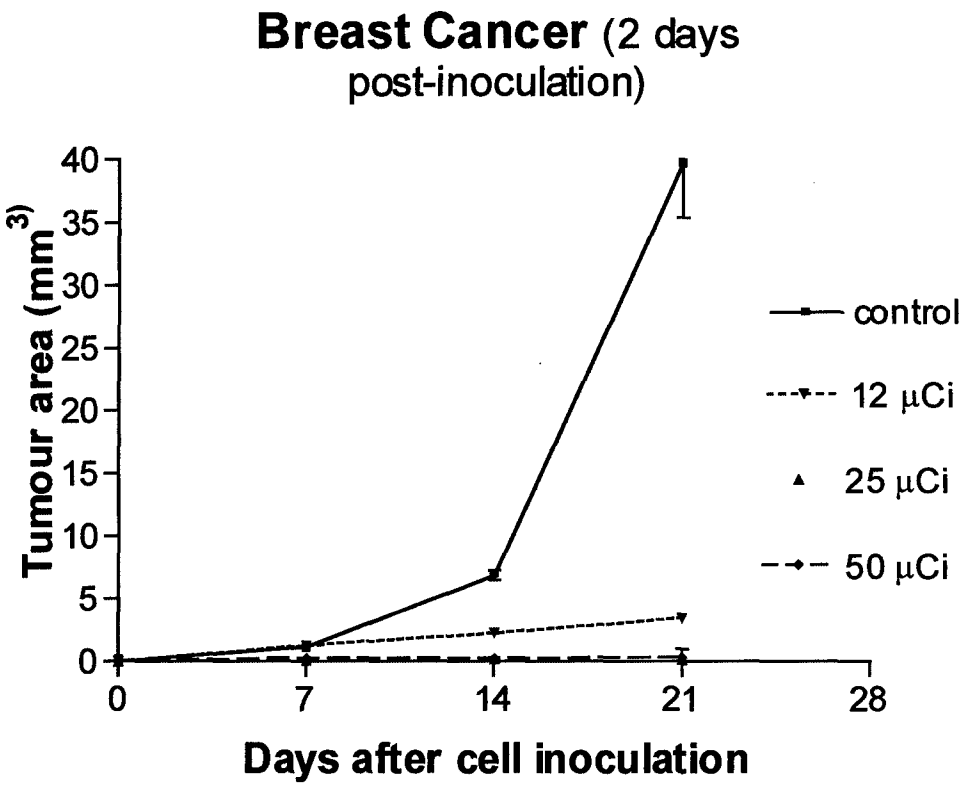


Figure 8 α-PAI2 and non-specific (NS) α-monoclonal antibody at 2-days post-inoculation

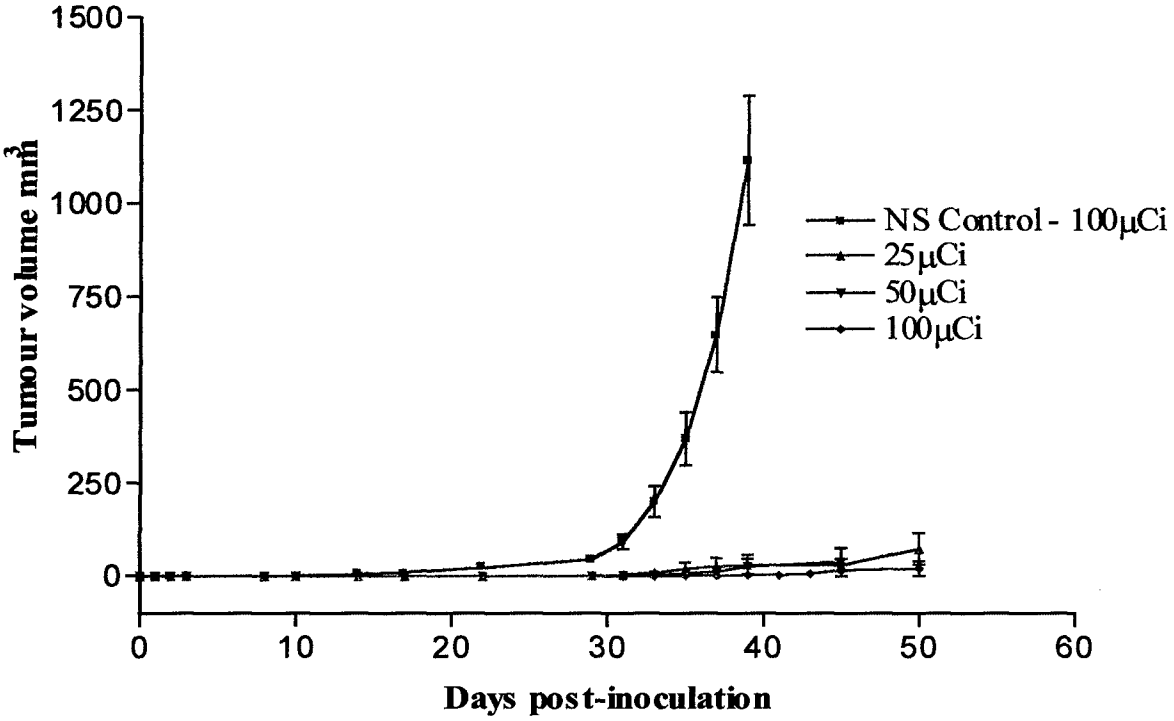
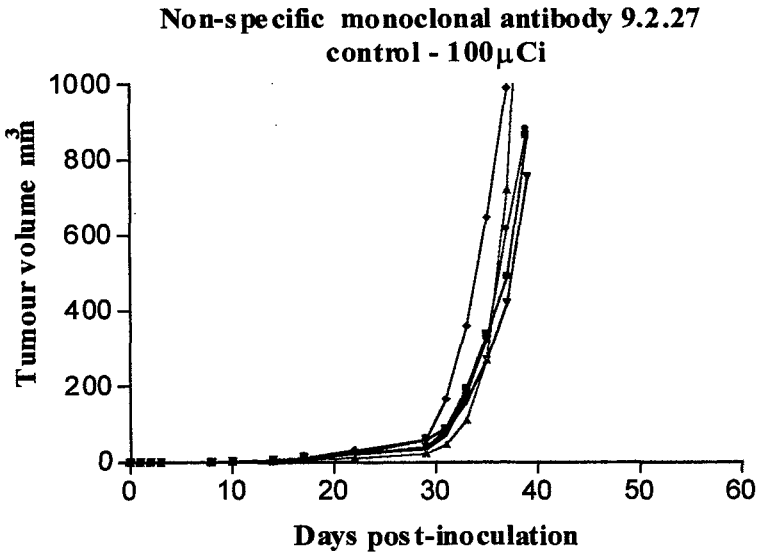
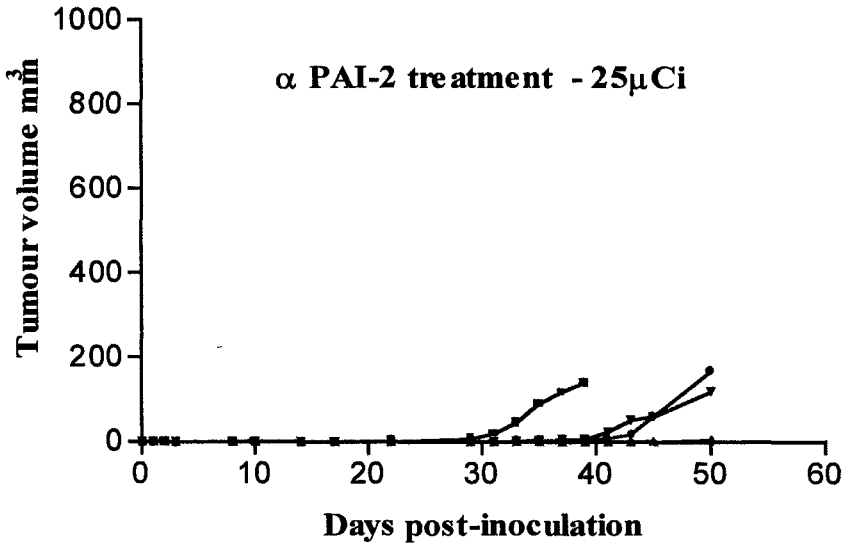
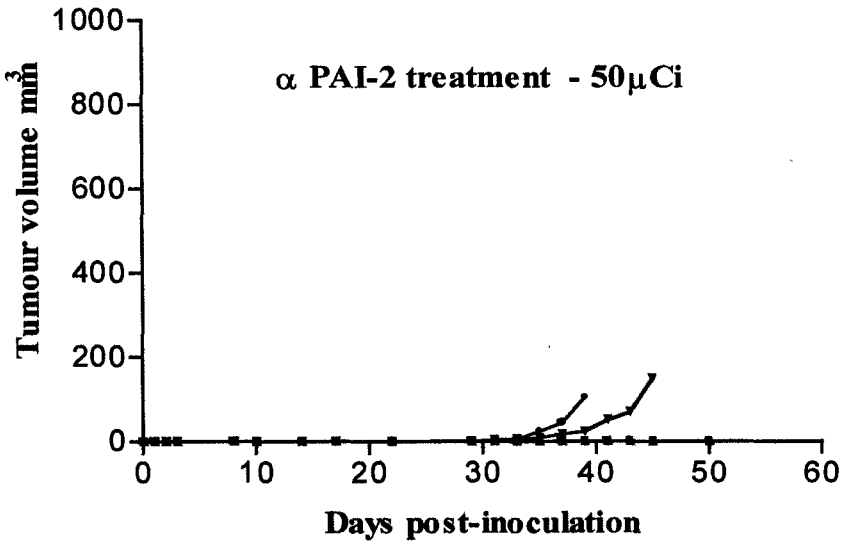


Figure 9 **Effect on tumour growth after systemic (ip) administration of alpha-PAI2 at 2-days post-inoculation of MDA breast cancer cells, showing an indicative dose response, with 4/5 tumours being controlled at 6mCi/kg**







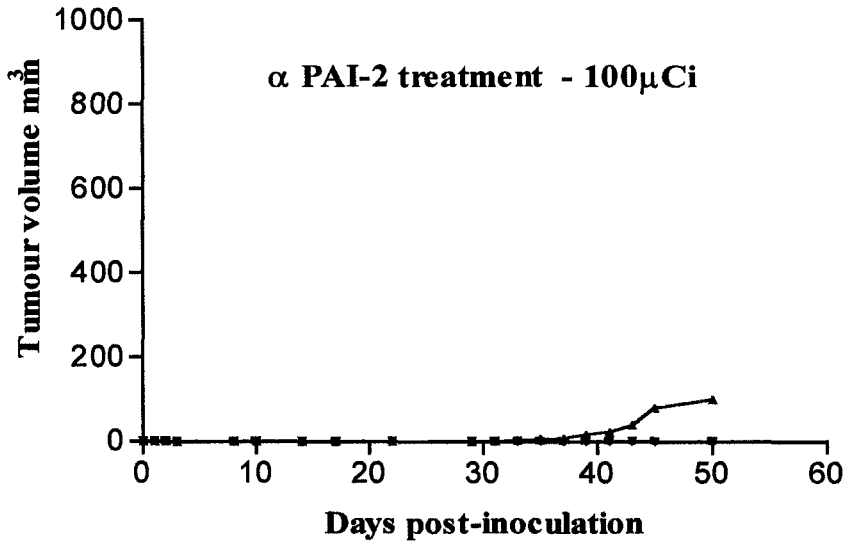
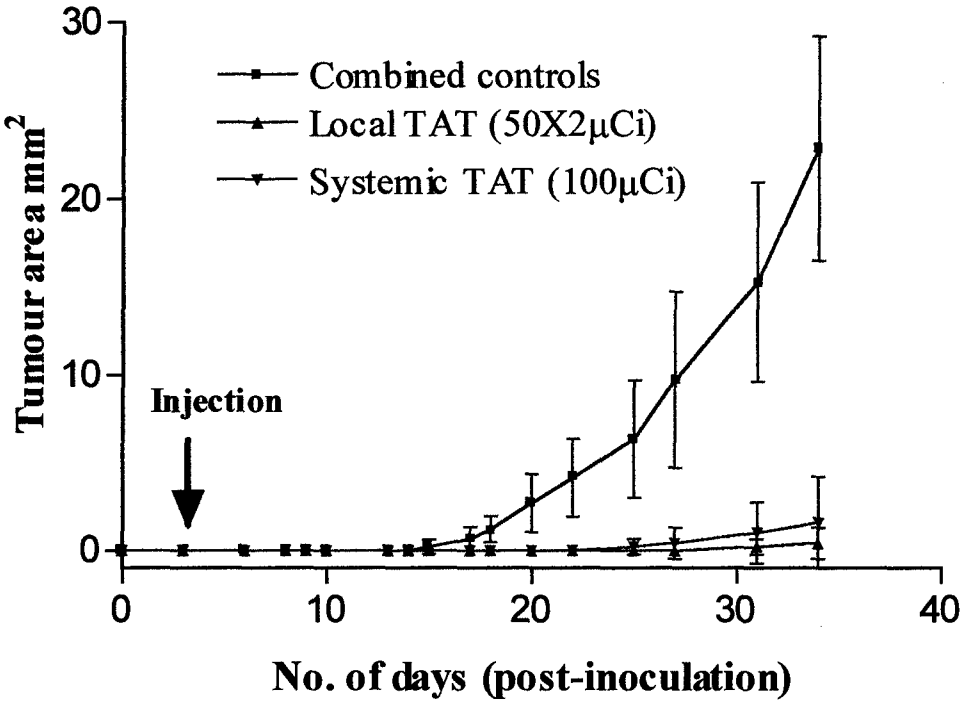


Figure 10 Efficacy of alpha-PAI2 for local and systemic inhibition of pre-angiogenic breast cancer lesions in the 2 day inoculation nude mouse model. The control curve (□) includes averaged results of tumour area after local and systemic injections of PBS and ^{213}Bi-BSA, which are not significantly different. For local TAT (Δ), bilateral injections of 50 μCi alpha-PAI2 are made, whereas for systemic TAT (∇), a single ip injection of 100 μCi is made.



KEY RESEARCH ACCOMPLISHMENTS

1 Mass spectroscopy measurements show multiple chelation to PAI2.
2 Stability of alpha-PAI2 determined in serum and for DTPA challenge.
3 In vitro targeted cytotoxicity demonstrated.
4 In vivo toxicity measurements show that therapeutic doses of alpha PAI2 up to 6 mCi/kg bodyweight can be delivered to mice without significant longer-term weight loss.
5 Biodistribution of alpha-PAI2 shows enhanced uptake in kidneys.
6 Preangiogenic status of the 2-day tumour model.
7 Complete inhibition of the growth of breast cancer tumours has been achieved by local alpha-PAI2 injection at 2 days post-inoculation.
8 Inhibition of tumour growth established for single systemic (ip) injection of alpha-PAI2.
9 Indicative dose response for systemic treatment with alpha-PAI2.
10 Non-specific ^{213}Bi-BSA does not cause inhibition of tumour growth.
11 In vitro results published in journal of Breast Cancer Research and Treatment.
12 Registration of international patent for alpha-PAI2.
13 Final in vivo paper submitted to British J Cancer for publication.

REPORTABLE OUTCOMES

Presentations:

Preclinical Studies Of Targeted Alpha Therapy For Human Breast Cancer Using ^{213}bi-Labeled-Plasminogen Activator Inhibitor Type 2. B J Allen, Z Tian, S Rizvi, Y Li, M Ranson. Breast cancer 2001: emerging possibilities. Melbourne, March 2001

In Vitro And Preclinical Studies Of Targeted Alpha Therapy For Human Breast Cancer Using ^{213}bi-Labeled-Plasminogen Activator Inhibitor Type 2. B J Allen, Z Tian, S Rizvi, Y Li, M Ranson. ANZSNM May 2001, Hobart

Manuscripts:

Allen BJ, Rizvi S, Li Y, Tian Z & Ranson M. (2001) In vitro and preclinical targeted alpha therapy for melanoma, breast, prostate and colorectal cancers. Critical Reviews in Oncology/Hematology. 39:39-146

Ranson M, Tian Z, Andronicos NM, Rizvi S, and Allen BJ (2002). In vitro cytotoxicity study of human breast cancer cells using Bi-213 labeled plasminogen activator type 2. M Ranson, Z Tian, N M Andronicos, S Rizvi, B J Allen. Breast Cancer Research and Treatment, 71; 2,149-159.

Manuscript submitted:

B J Allen, S M A Rizvi, Y Li, Z Tian: "Preclinical studies of targeted alpha therapy for breast cancer using ^{213}Bi-labeled plasminogen activator type 2". Submitted to British J Cancer.

Patent applied for and/or issued:

Australian provisional patent number PQ5824, file date 24/2/2000, "PAI-2 conjugates for the treatment and imaging of cancer". Filed in the name of Biotech Australia Pty Ltd, University of Wollongong, Medical Scitec Australia Pty Ltd. Full international patent is being registered, PCT-AU01-00185.

Funding applied for based on work supported by this award:

USAMRMC CTR Grant Application for 2002-4: "Development and implementation of alpha-PAI2 for targeted alpha therapy of breast cancer."
Principle Investigators: B J Allen (St George Hospital & University of Wollongong), M Ranson (University of Wollongong), M Links (St George Hospital), C L Bunn (Biotech Australia).
This was a successful pre-proposal BC014016, but not funded.

USAMRMC Grant Application for 2002-4: "Preclinical trials of multi-targeted alpha therapy for the control of micrometastatic prostate cancer". B J Allen (St George Hospital), P J Russell (Prince of Wales Hospital), M Ranson (University of Wollongong), P Cozzi (St George Hospital).
Not funded.

NHMRC Grant Application 2002-3: Targeted alpha therapy: development of a new treatment for metastatic cancer. B J Allen, M Ranson, C Bunn, M Links. ID 213119.
Funded for two years.

USAMRMC Grant Application for 2003-5: "Preclinical study of multiple targeted alpha therapy for the control of micrometastatic prostate cancer. Y Li (St George Hospital). New Investigator Award PC020377.

CONCLUSIONS

The outcomes of this research provide a strong case for the therapeutic use of alpha-PAI2, where the targeting protein, PAI2, adheres to breast cancers cells and the alpha emitter Bi-213, kills these targeted cells. As such, we are clearly moving towards the development of a new therapeutic modality for breast cancer.

If ultimately successful, alpha-PAI2 therapy could change the prognosis for many breast cancer patients who are clinically free of disease but who have a high probability of micrometastatic disease that would eventually lead to a reduced life span. The next step is the investigation of sequential dose treatment model as a prelude to clinical application.

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APPENDICES

1 Abstract: In vitro and preclinical studies of targeted alpha therapy for human breast cancer using ^{213}bi-labeled-plasminogen activator inhibitor type 2 .

2 Paper by Ranson et al, Breast Cancer Research and Treatment.

IN VITRO AND PRECLINICAL STUDIES OF TARGETED ALPHA THERAPY FOR HUMAN BREAST CANCER USING ^{213}BI-LABELED-PLASMINOGEN ACTIVATOR INHIBITOR TYPE 2

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Abstract

Many breast cancer patients receive minimal long-term survival benefit despite undergoing surgery and adjuvant therapy, indicating that micrometastases are not eradicated. We are developing a new adjuvant therapy for the control of metastatic breast cancer based on alpha-emitting nuclides. This therapy exploits the involvement of cell-surface receptor bound urokinase plasminogen activator (uPA) in the metastatic spread of breast cancer cells. Once bound to specific cell-surface receptors, uPA efficiently activates plasminogen to the broad-spectrum protease, plasmin. A large body of experimental and clinical evidence implicates the uPA system as a marker of malignancy and therefore a useful and accessible specific target for therapy.

We have successfully labeled and tested recombinant human PAI2 with the alpha radioisotope ^{213}Bi to produce alpha-PAI2. Low doses of alpha-PAI2 are highly cytotoxic towards breast cancer cell lines in vitro, whereas non-specific alpha-BSA had no cytotoxic effect, reflecting that non-targeted cells are immune from alpha-PAI2.

In vivo toxicity studies in nude mice show that up to 6 mCi/kg of alpha-PAI2 (ip) is well tolerated. In vivo efficacy experiments in nude mice demonstrate in 5/5 mice that a local injection of alpha-PAI2 can completely inhibit the growth of tumour at 2 days post-cell inoculation. Further, systemic (iv) administration of alpha-PAI2 at 2 days post-inoculation can also cause tumour growth inhibition in a dose dependent manner, with 3/5 tumours uncontrolled at 1.5 mCi/kg, 2/5 at 3 mCi/kg and 1/5 at 6 mCi/kg. Thus alpha-PAI2 is successful in targeting isolated cells and preangiogenic cell clusters.

These results indicate the promising potential of alpha-PAI2 as a novel therapeutic agent for micrometastatic breast cancer.

Key words:

Bismuth-213 (^{213}Bi), breast cancer cells, plasminogen activator inhibitor type 2 (PAI2), urokinase, targeted alpha therapy.

Breast Cancer Research and Treatment **71:** 149–159, 2002.

Report

In vitro cytotoxicity of bismuth-213 (^{213}Bi)-labeled-plasminogen activator inhibitor type 2 (alpha-PAI-2) on human breast cancer cells

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Key words: bismuth-213 (^{213}Bi), breast cancer cells, plasminogen activator inhibitor type 2 (PAI-2), targeted alpha therapy, urokinase

Summary

Metastasis is the principal cause of death in breast cancer patients. New and improved treatments for eradicating micrometastases are needed. To this end, a novel alpha-emitting protein construct, ^{213}Bi-labelled plasminogen activator inhibitor type-2 (PAI-2) (alpha-PAI-2), was evaluated *in vitro*. This construct exploits: (a) the over-expression of the cell-surface receptor bound urokinase plasminogen activator (uPA) in the metastatic spread of breast cancer cells; (b) the binding and inhibition of receptor-bound uPA by PAI-2; and (c) the high cytotoxicity of alpha radiation. High labeling efficiencies and stability of ^{213}Bi bound to human recombinant PAI-2 conjugated with cyclic diethylenetriaminepentaacetic acid anhydride were achieved (greater than 90%). The uPA inhibitory activity of the chelated PAI-2 was maintained as determined by complex formation with uPA and by inhibition of uPA activity. Furthermore, the reactivity of alpha-PAI-2 was confirmed in a cell assay as this construct was highly cytotoxic to breast cancer cell lines that express active, receptor bound uPA. The specificity of alpha-PAI-2 targeting was shown using several controls. Firstly, an active uPA blocking agent that limits PAI-2 binding significantly improved cell survival by a factor greater than three. Secondly, a non-specific alpha-BSA construct had minimal cytotoxic effect. Moreover, alpha-PAI-2 was not cytotoxic to freshly isolated normal human leukocytes, confirming that cells which do not contain active, receptor bound uPA cannot be targeted by alpha-PAI-2. In conclusion, we have validated, *in vitro*, the potential of alpha-PAI-2 as a novel therapeutic agent for breast cancer.

Introduction

The major failure in breast cancer management is due to the incomplete killing of malignant tumour cells that have spread throughout the body [1]. This is despite the many treatments available, such as surgery, radiation therapy, hormone therapy and chemotherapy. The American Cancer Society estimated 182,800 new cases of invasive breast cancer in the year 2000 among women in America, and 40,800 are expected to die from the disease [2]. Novel, more effective treatments that overcome this problem in breast cancer management are essential. Targeted therapy, first discussed over 100 years ago, is based on the idea that a drug will attack its target without damaging other tissues [3]. Targeted alpha therapy (TAT) uses an alpha emitting radionuclide as a lethal medicament via an effective targeting carrier to kill cancer cells [4]. We are investigating a novel TAT approach that exploits the involvement of cell-surface receptor bound urokinase plasminogen activator (uPA) in the metastatic spread of breast cancer cells.

Alpha emitting radionuclides emit alpha particles with energies up to an order of magnitude greater than most beta rays, yet their ranges are two orders of magnitude less as alpha particles have a linear energy transfer (LET) which is about 100 times greater [5]. This is manifested by a high relative biological effectiveness (RBE). As a result, a much greater fraction of the total energy is deposited in cells with alphas and

very few nuclear hits are required to kill a cell. Consequently, a 100-fold enhancement in radiation dose [6, 7] would be delivered to the nucleus of a cancer cell if an effective carrier were employed to take the alpha radionuclide into that cancer cell. Thus, only alphas have the possibility to kill the metastatic cancer cells at tolerable dose limits, whereas the low LET of betas make this a very difficult task within human dose tolerance limits.

Availability of the alpha emitting radionuclides has been the major problem in the past for their large-scale scientific and clinical application. Studies have been carried out on ^{211}At and ^{212}Bi [8–11] with encouraging results. The stable and reliable ^{225}Ac generator of the alpha emitting nuclide ^{213}Bi has been produced, modified and used successfully [11–22], with several of these studies indicating a therapeutic potential of ^{213}Bi-labeled antibody constructs against tumour cells both *in vitro* and *in vivo*. Our group has modified methods of conjugating ^{213}Bi radionuclide to antibodies with the stable chelator cyclic diethylenetriaminepentacetic acid anhydride (cDTPA) for use in the alpha therapy of melanoma [18], colorectal cancer [21] and leukaemia [22].

A large body of experimental and clinical evidence implicates over-expression of the urokinase plasminogen activator (uPA) system as a modulator of the aggressive behaviour of tumour cells and as a strong prognostic factor for predicting poor breast cancer patient outcome [23–25]. uPA converts plasminogen into the highly active protease plasmin [23]. Plasmin promotes tissue degradation and remodelling of the local extracellular environment by directly and indirectly (via activation of pro-metalloproteases) degrading extracellular matrix molecules [23–25]. uPA is synthesised and secreted as a pro-enzyme, whose activation is markedly accelerated upon binding with high affinity (0.1–1 nM) to specific cell surface uPA receptors (uPAR) [23, 26]. Receptor density varies depending on cell type (10^3–10^6 sites/cell) [26].

The broad substrate specificity of plasmin requires strict regulation at many levels to limit activation to only physiologically appropriate times and sites. Occupancy of uPAR with active uPA is an important requirement in rapidly generating cell-surface plasmin [23, 24, 26] allowing pericellular proteolysis that facilitates cellular migration and invasion. Since plasma uPA concentrations are very low (~20 pM) [24], this suggests that efficient cellular uPAR occupancy results only from autocrine and/or paracrine synthesis and secretion of uPA. Synthesis of uPA, and uPAR are subject to regulation by local or systemic signals, expressed appropriately as in the case of inflammation, angiogenesis, or wound healing, or inappropriately as in the case of tumour invasion [23, 24]. The fact that normal, quiescent tissues express little or undetectable uPA (e.g., brain and blood [27, 28] also see below) suggests that these tissues would have largely unoccupied uPAR, if indeed they express uPAR at all, as would be expected for cells that are not invasive.

Immunohistochemical and *in situ* hybridisation studies have localised uPA and uPAR to various tumour and associated stromal cells [24]. The strongest and most consistent expression universally occurs in primary carcinoma tissue with high tumour grade, and in some cases has been further localised to cells at the invasive margins. For example, 79–90% [29], 83% [30] and 100% [31–34] of invasive ductal carcinomas were positive for uPA antigen or mRNA, compared to 28–100% of non invasive cases [29, 30, 32, 33]. In contrast, benign lesions such as fibroadenoma and normal breast tissue were either negative or with some occasional weak/diffuse staining for all markers in the majority of studies that included such tissue for comparison. Clinical data from multiple, independent groups measuring activity and/or antigen levels in tumour extracts confirm a relationship between high levels of uPA in primary breast tumour (e.g., > 10 fold more than in normal breast [26], and a poor relapse-free, and/or overall survival [reviewed in 24, 25].

The activity of uPA is physiologically regulated by plasminogen activator inhibitors type 1 and 2 (PAI-1 and PAI-2) [23, 24, 35]. PAI-2, a member of the serpin (serine proteinase inhibitor) family, is produced by many cell types as either a predominantly intracellular non-glycosylated form (43–47 kDa) or as an extracellular glycosylated form (60 kDa) [35]. Unlike many other serpins both PAI-2 forms are insensitive to oxidative inactivation [35]. PAI-2 is an efficient inhibitor of active uPA forming SDS-stable 1 : 1 complexes with uPA (rate constant 10^6 $M^{-1}S^{-1}$) [35]. Cell surface-bound uPA is accessible to and inhibitable by exogenous PAI-2 [35, 36], and a number of studies have suggested the potential for PAI-2 to inhibit cancer cell invasion and metastasis [35]. The pharmacokinetics and biodistribution of human recombinant ^{125}I-labelled PAI-2 in both control mice and mice bearing human colon cancer (uPA-positive HCT116 cell line) xenografts has been established [36]. Briefly, ^{125}I-PAI-2 localised in 0.5 cm^3 tumour xenografts quickly (after 1 min, peaking at 30–60 min at approx. 1.5%

of total injected dose). Furthermore, repeat intravenous, sub-cutaneous, or intra-peritoneal injections of ^{125}I-PAI-2 resulted in an accumulation of radioactivity without an accompanying increase in the major organs or in toxicity. In addition, tumour associated ^{125}I-PAI-2 correlated with tumour mass. Such studies indicate that invasive and metastatic tumour cells, shown consistently to contain active uPA, would be accessible to and targeted by exogenously administered PAI-2.

It is clear that uPA is a specific marker of malignancy and that PAI-2 represents a useful targeting agent. In this paper we report for the first time, the production and evaluation of a new alpha-nuclide emitting cytotoxic agent ^{213}Bi-labeled PAI-2 (alpha-PAI-2). The reactivity and specificity of alpha-PAI-2 cytotoxicity for two human breast cancer cell lines *in vitro* holds promise for alpha-PAI-2 as a new therapeutic modality for the control of tumour metastases.

Materials and methods

Materials

Human recombinant PAI-2 (47 kDa) was provided by Biotech Australia Pty Ltd. Glu-plasminogen was purified from human plasma as described by Andronicos et al. [37]. Microspin concentrators were purchased from Millipore (Bedford, MA, USA). RPMI-1640 was purchased from Life Technologies (Castle Hill, NSW, Australia). Fetal calf serum (FCS) was obtained from Trace Bioscientific (Castle Hill, NSW, Australia). The cyclic anhydride of diethylenetriaminepentacetic acid (cDTPA) was purchased from Aldrich Chemical Company. Bovine serum albumin (fraction V) (BSA) and propidium iodide (PI) were purchased from Sigma Chemical (St Louis, MO. USA). Human twin chain urokinase plasminogen activator (tc-uPA) was purchased from Serono (Sydney, NSW, Australia). Glu-gly-arg chloromethylketone (EGR-CMK) was purchased from Calbiochem (Sydney, NSW, Australia). Spectrozyme-UK (carobenzoxy-L-γ-glutamyl-(α-t-butoxy)-glycyl-arginine-p-nitroanilide-diacetate), mouse anti-human uPA IgG_1 (#394), mouse anti-human uPAR IgG_{2a} (#3696), and mouse anti-human PAI-2 IgG_1 (#3750) monoclonal antibodies were purchased from American Diagnostica Inc (Greenwich, CT, USA). Mouse isotype control subclasses IgG_1, IgG_{2a} antibodies and fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG were from Silenus (Sydney, NSW, Australia). Fresh human leukocytes were isolated from whole blood using Fycoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions.

Radioisotope

Alpha particle emitting radionuclide, ^{213}Bi, was produced from the ^{225}Ac/^{213}Bi generator which was purchased from the Oak Ridge National Laboratory, United States Department of Energy. ^{213}Bi was eluted from the ^{225}Ac column with 250 μl of freshly prepared 0.15 M distilled and stabilized hydriodic acid followed by washing with 250 μl sterile distilled water [38]. The first elution was not used, and a time of 2–3 h was allowed for ^{213}Bi to regenerate on the column for the next elution. For all activity calculations corrections were made for ^{213}Bi decay using the half-life of 45 min.

PAI-2 conjugation with cDTPA, stoichiometry and reactivity

PAI-2 and BSA were conjugated with cDTPA by a modification of the method described by Boll et al. [38] and Paik et al. [39], to give the desired protein-DTTA conjugate. PAI-2/BSA (1 mg) dissolved in PBS was conjugated to cDTPA by first increasing the pH to approximately 8.2 via the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9.0). A 50-fold molar excess of cDTPA (found to result in smallest fraction of underivatised PAI-2; data not shown) was added and the mixture incubated at 25°C for 1 h with intermittent rocking. The reaction was stopped with a final concentration of 10% (v/v) 1 M Tris–HCl (pH 7.2). The final reaction volume was 0.5 ml. Three reaction volumes of PBS were used to purify DTTA-labeled proteins away from free cDTPA using a microspin concentrator as described by the manufacturer.

The concentrations of the protein-DTTA conjugates were measured by BIORAD DC protein assay reagent kit. The stoichiometry of DTTA-PAI-2 was determined using electrospray ionisation mass spectrometry as previously described [40]. For this purpose, the DTTA-PAI-2 was completely desalted into MilliQ water prior to analysis. The uPA binding activity of PAI-2 or DTTA-PAI-2 conjugate was examined by their ability to form complexes after 90 min incubation with equimolar amounts of tc-uPA at 37°C [36]. The uPA:PAI-2 complexes were detected by SDS-PAGE (12% non-reducing gel). The uPA enzymatic inhibitory activity of PAI-2 or DTTA-PAI-2 conjugate was measured as previously described [37]. Briefly, after

incubation of PAI-2 or DTTA-PAI-2 for 30 min at 37°C with equimolar ratios of tc-uPA, 2 mM of the uPA chromogenic substrate (Spectrozyme-UK) was added and the absorbance in each well was recorded over 10 min at 405 nm using a SPECTROmax plate reader (Molecular Dynamics). Standard curves were constructed using tc-uPA (0–20 IU/well).

^{213}Bi labeling of DTTA-PAI-2

Concentrated DTTA-PAI-2 stocks were diluted with 500 mM sodium acetate at pH 5.5 and 5–10 μg of DTTA-PAI-2 was labeled with free ^{213}Bi for 20 min at room temperature. After labeling, ^{213}Bi-DTTA-PAI-2 (alpha-PAI-2) was buffer exchanged into PBS using a PD-10 column using PBS (pH 7.0) as the eluting buffer. ^{213}Bi-DTTA-BSA (alpha-BSA) was radiolabeled by similar methods. The radiolabeling efficiency was determined by Instant Thin Layer Chromatography (ITLC) using a 10 μl aliquot of the final reaction mixture applied to Gelman paper (strip size 1 × 9 cm, Gelman Science, Ann Arbor, MI). The paper strips were developed using 0.5 M sodium acetate (pH 5.5) as the solvent. The paper strips were cut into four sections and the gamma emissions from the radioisotope in each section was counted using a 340–540 keV window. The radiolabeled protein is found at the origin section, while free radioisotope is found at the solvent front section.

The stability of ^{213}Bi labeled protein conjugates in human serum was determined by incubation in fresh human serum at 37°C. At 0 and 45 min, 10 μl samples were withdrawn and subjected to ITLC. The origin activity reflects the stability of radiolabeled protein conjugates. The solvent front activity reflects the leaching of radiolabeled protein conjugates.

Cell culture

The metastatic MDA-MB-231 and non-metastatic MCF-7 human breast cancer cell lines [41] were routinely cultured in RPMI-1640 supplemented with 10% (v/v) heat-inactivated FCS and passaged using Trypsin/EDTA. The cells were incubated in a humidified incubator at 37°C with a 5% carbon dioxide air atmosphere. For all experimental procedures, subconfluent cells that had been in culture for 48 h without a change of media were harvested by rinsing flasks twice with PBS (pH 7.2) and then detaching with PBS/0.5 mM EDTA at 37°C for 5 min. Cells were collected and resuspended in the appropriate buffer as described below.

Flow cytometry

For the detection of cell-surface uPA, uPAR and PAI-2 indirect immunofluorescence staining was performed as described by Ranson et al. [41]. Briefly, cells were incubated with either an irrelevant isotype control or anti-human uPA, uPAR or PAI-2 monoclonal antibody for 30 min on ice (10 μg ml^{-1} in cold RPMI/0.1% BSA). The cells were washed with 1 ml of cold RPMI/0.1% BSA and incubated with FITC-conjugated anti-mouse IgG (1 : 50 dilution of stock in cold RPMI/0.1% BSA) for 30 min on ice in dark. The cells were washed again and resuspended in 0.5 ml cold PBS/0.1% sodium azide containing 5 μg ml^{-1} propidium iodide (PI). Cell-associated fluorescence was then measured by dual-colour flow cytometry. This technique was used to establish 'gates' – the exclusion of PI for a viable 'gate', the inclusion of PI for a non-viable 'gate' [41].

All flow cytometry data were analysed using CELLQuest software (Becton-Dickinson). Isotype control fluorescences were subtracted from all flow cytometry experiments. Only cell-surface associated (viable) cell fluorescence is reported.

Immunohistochemistry

Cells grown on glass coverslips for 48 h in growth medium, were washed three times with PBS at room temperature, fixed by incubation with PBS supplemented with 1% gluteraldehyde for 1 h at room temperature, and then washed once with PBS. Endogenous peroxidases were inactivated with 3% H_2O_2 for 5 min at room temperature. The cells were incubated with 10% human serum for 30 min, washed with PBS, and then incubated for 1 h with primary antibodies diluted with PBS/10% human serum (final concentration 20 μg/ml) at room temperature. The cells were washed twice with PBS and incubated with the secondary reagents from the DAKO LSAB+ Kit according to the manufacturer's instructions. After rinsing with PBS, the cells were viewed using a video camera (National Panasonic) attached to an inverted compound microscope (Leica, Germany). Images of the cells (original magnification x400 unless otherwise stated) were captured by a Power PC (Macintosh 8500/20) using Apple Video Player software (Macintosh).

Cytotoxicity assay

The CellTiter 96 Aqueous non-radioactive cell proliferation assay (Promega, WI, USA) was used to

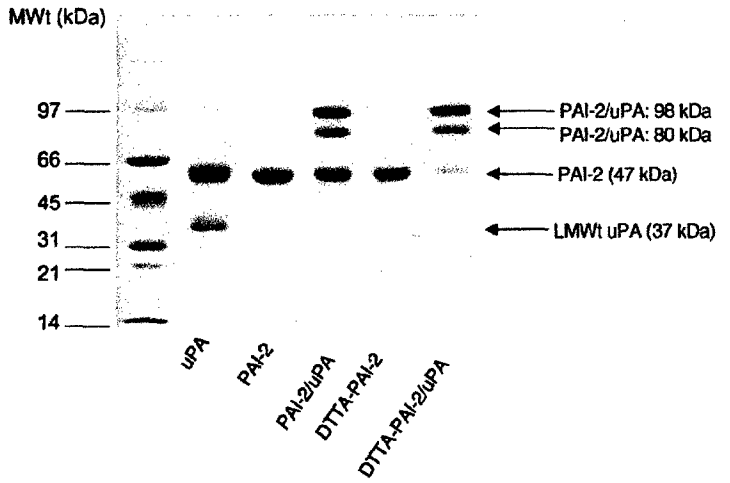

Figure 1. Coomassie stained 12% SDS-PAGE showing complex formation between high and low molecular weight tc-uPA and PAI-2 or DTTA-PAI-2. Complexes were formed by incubating either labeled or unlabeled PAI-2 with equimolar amounts of tc-uPA for 1.5 h at 37°C. The reaction was terminated by adding 5-times non-reducing SDS-PAGE sample buffer (20% v/v) to the reaction mix. LMWt corresponds to low molecular weight uPA that retains enzymatic activity of native uPA.

determine the effect of ^{213}Bi labeled proteins on cell survival.

The activities of freshly labeled alpha-PAI-2 and alpha-BSA preparations were measured using a radioisotope calibrator and neutralized to pH 7.0 via the addition of 10% (v/v) 1 M $NaHCO_3$ (pH 9.0). Immediately after this, five serial doses of alpha-PAI-2 and one dose of alpha-BSA were prepared in 100 µl RPMI/10% FCS and added to 96-well plates in triplicate containing 20,000 cells/well in 100 µl RPMI medium/10% FCS. The plates were then incubated overnight in a 5% carbon dioxide atmosphere at 37°C. Controls were performed in triplicate in the same 96-well plate for each experiment and consisted of RPMI/10% FCS medium alone. In some cases further controls were performed as described in Table 1. In all cases the cells had been previously pre-incubated with 20 µg/ml plasminogen for 20 min at room temperature and washed before placing into 96-well plates. In some cases, the cells were also treated with 0.5 mM EGR-CMK (a specific uPA inhibitor) [36] for 15 min at room temperature after plasminogen activation and prior to incubation with radiolabeled proteins.

The cells were then washed and incubated with 100 µl phenol-red free RPMI without FCS containing 20 µl of the CellTiter 96 reagent. After 3 h incubation in a 5% carbon dioxide atmosphere at 37°C, the reaction was stopped by the addition of 10% SDS, and the absorbance in each well was recorded at 490 nm using a SPECTROmax plate reader. The absorbance reflects the number of surviving cells. Blanks were subtracted from all data and analysed using Prism software (GraphPad Software Inc, USA).

Results

DTTA-PAI-2 retains its uPA inhibitory activity

PAI-2 conjugated with a 50-fold molar excess of cDTPA resulted in preparations with stoichiometries ranging from 1–4 DTTA molecules per molecule of PAI-2, as determined by the mass spectrometry method [40]. Importantly, less than 3% of PAI-2 was underivatised in these preparations and PAI-2 dimers, due to DTTA-mediated intermolecular crosslinking, were not observed. This indicates that under the conjugation conditions used in this study, almost the entire PAI-2 preparation consisted of DTTA-PAI-2.

The incubation of uPA with PAI-2 in a 1 : 1 molar ratio forms a SDS-stable uPA-PAI-2 complex that is indicative of the inhibitory activity of the molecule [36]. Thus, SDS-PAGE can be used to determine if the uPA inhibitory activity of DTTA-PAI-2 was retained. As demonstrated in Figure 1 both unlabeled PAI-2 and DTTA-PAI-2 were able to form SDS-stable complexes with both high molecular weight tc-uPA (98 kDa) and active low molecular weight uPA (80 kDa). Quantita-

tively, the uPA inhibitory capacity was determined using the Spectrozyme-UK assay. No significant difference was observed between the uPA inhibitory activity of DTTA-PAI-2 and unmodified PAI-2 since both resulted in >98% inhibition of uPA enzymatic activity when 1 : 1 molar ratios were used (data not shown). Thus, the conjugation of PAI-2 with cDTPA did not significantly perturb the ability of PAI-2 to bind to and inhibit the activity of uPA.

^{213}Bi radiolabeling of DTTA-PAI-2 (alpha-PAI-2)

The radiolabeling reaction efficiency of alpha-PAI-2 was determined by ITLC (Figure 2(A)). Free isotope ^{213}Bi standard migrates to the solvent front of the ITLC strip (the 4th fraction). In contrast, greater than 90% of the radioactivity associated with alpha-PAI-2 was found at the origin (the 1st fraction) indicating a high efficiency of labeling. The specific activity of the alpha-PAI-2 was approximately 10–15 μCi/μg.

When incubated in human serum for 45 min (half-life of ^{213}Bi), less than 10% of the label leached from the alpha-PAI-2 sample as determined by the amount of radioactivity in the 4th fraction compared to the total radioactivity (Figure 2(B)). This indicates that *in vitro* the labeled protein is stable in serum.

Cell surface expression of endogenous uPA, uPAR and PAI-2

The characteristic high and low cell surface levels of endogenous uPA and uPAR expression on MDA-MB-231 and MCF-7 cells, respectively [41], were confirmed by dual colour flow cytometry (Figure 3(A)) and immunohistochemistry (Figure 3(B)). The endogenous cell surface levels of PAI-2 were also examined on these cell lines by flow cytometry (Figure 3(A)). While the uPA and uPAR antigen levels were approximately 28-fold and 6-fold higher on the metastatic MDA-MB-231 cells compared to the non-metastatic MCF-7 cells, the endogenous levels of cell surface associated PAI-2 were comparably very low on both cell lines (Figure 3(A)). Despite the low cell surface PAI-2 levels, both cell lines produce intracellular PAI-2 as detected by western blotting of whole cell lysates (data not shown). These results suggest that cell-surface bound uPA is not appreciably complexed with endogenously produced PAI-2.

Immunohistochemistry mirrored the endogenous uPA and uPAR antigen expression differences seen in the two cell lines by flow cytometry (Figure 3(B)). Notably, the staining patterns of both antigens for both cell lines were punctuate and heterogeneous. Most of the MDA-MB-231 cells were highly positive for either antigen with less than 10% being weakly positive. In contrast, most of the MCF-7 cells were only weakly positive with less than 10% being moderately positive.

Cytotoxicity of alpha-PAI-2 towards the MDA-MB-231 and MCF-7 cell lines

Alpha-PAI-2 was found to be highly toxic to MDA-MB-231 and MCF-7 cells (Figure 4, Table 1). In contrast, alpha-BSA showed only slight toxicity compared with alpha-PAI-2 at the maximum activity used. No significant toxicity was observed with either DTTA-PAI-2 or PAI-2 (Table 1). The D_o (37% cell survival) values with alpha-PAI-2 were calculated to be 2.1 ± 0.2 μCi ($n = 6$) and 2.3 ± 0.2 μCi ($n = 6$) for MDA-MB-231 and MCF-7 cell lines, respectively. Notably, the D_o values of cells pre-treated with uPA specific inhibitor EGR-CMK prior to incubation with alpha-PAI-2 increased to 5.3 ± 0.2 μCi ($n = 2$) and 5.1 ± 0.2 μCi ($n = 2$) for MDA-MB-231 and MCF-7, respectively. At the maximum dose of alpha-PAI-2 (5 μCi) cell survival was reduced to 11–13% for both cell lines. In comparison, at this maximum dose of alpha-PAI-2 the survival of EGR-CMK pre-treated cells increased by a factor of 3.3 (Table 1). Furthermore, freshly isolated normal human leukocytes, which have undetectable levels of cell-surface bound active uPA (as determined by flow cytometry, data not shown) were unaffected by the maximum dose of alpha-PAI-2 in the absence or presence of EGR-CMK (Table 1).

An apparent difference in cell survival between the cell lines at dose concentrations below D_o (i.e., 2 μCi) in both the absence and presence of EGR-CMK (Figure 4) was observed. For example, at 0.625 μCi of alpha-PAI-2, $65 \pm 5\%$ versus $93 \pm 1\%$ of MDA-MB- 231 cells survived in the absence or presence of EGR-CMK, respectively. In comparison, $82 \pm 13\%$ versus $79 \pm 5\%$ of MCF-7 cells survived in the absence or presence of EGR-CMK, respectively. This suggests that at very low concentrations alpha-PAI-2 is more effective at killing MDA-MB-231 cells in a uPA-dependent manner.

Discussion

A number of novel techniques that target the uPA system for tumour therapy have been suggested and are

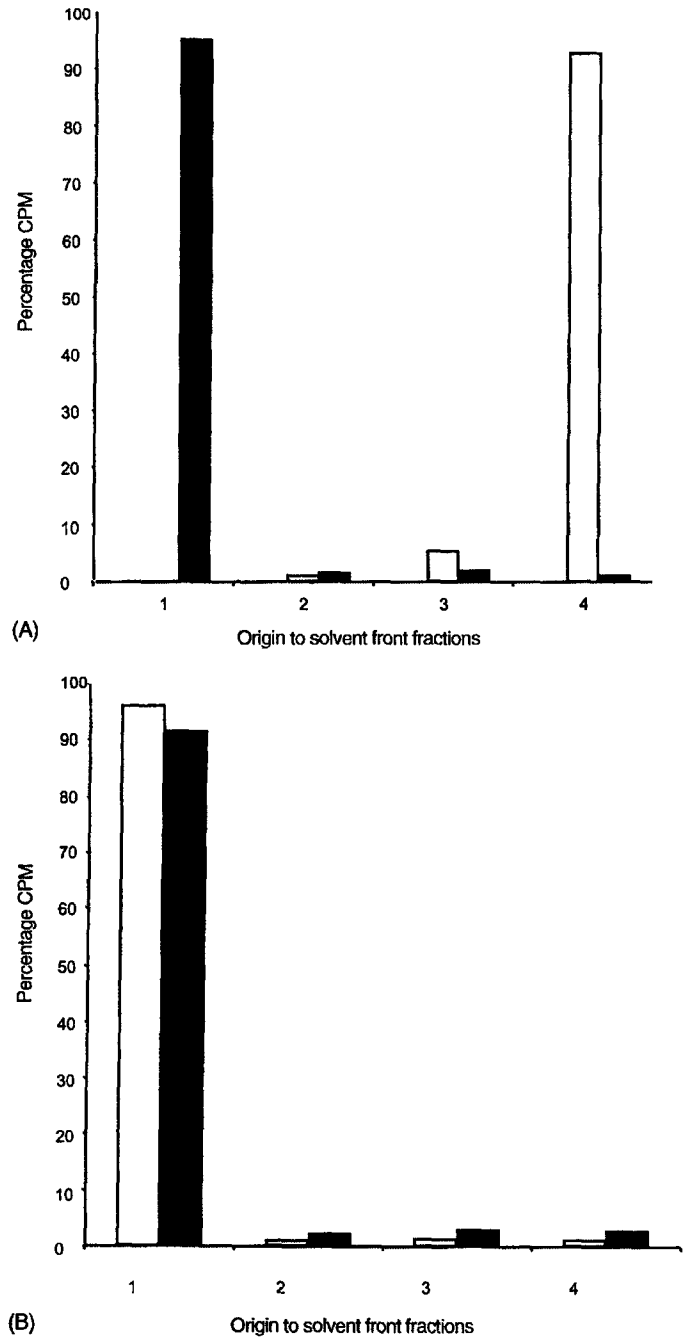

Figure 2. Analysis of ^{213}Bi labeled DTTA-PAI-2 (alpha-PAI-2). (A) Aliquots (10 µl) of freshly eluted alpha-PAI-2 (filled bars) or ^{213}Bi standard (unfilled bars) were spotted onto the origin of separate ITLC strips. After mobilization, the strips were cut into four pieces for gamma counting. Fraction 1 corresponds to the sample origin while fraction 4 corresponds to the solvent front. (B) Serum stability of alpha-PAI-2 incubated at 37°C in human serum for 0 min (unfilled bars) and 45 min (filled bars) and subjected to ITLC and analysed as per (A).

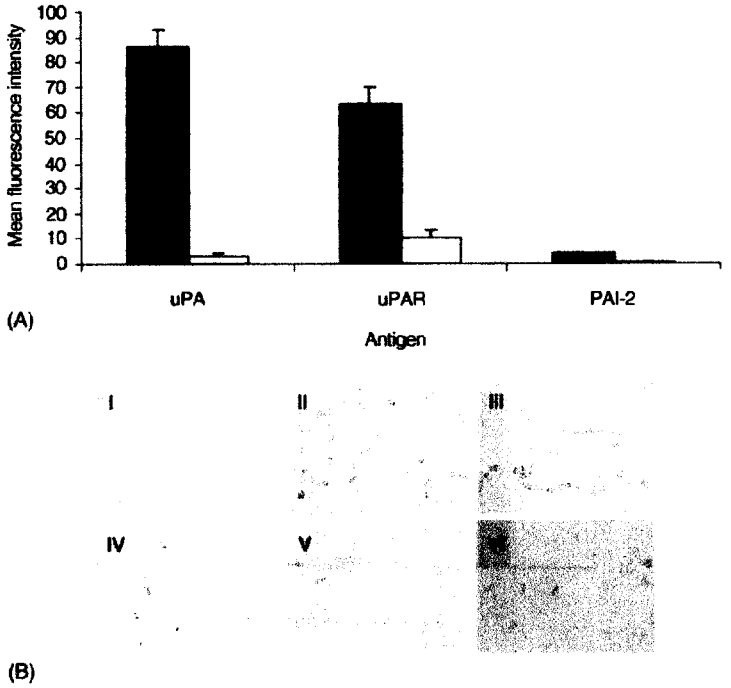

Figure 3. (A) Cell surface expression of endogenous uPA, uPAR and PAI-2. Viable MDA-MB-231 (filled bars) and MCF-7 (unfilled bars) cells were analysed for the specific expression of the various antigens by dual-colour flow cytometry as described in the methods. Values shown are means ±SD ($n > 3$). (B) Immunohistochemistry of fixed, non-permeabilised MDA-MB-231 (panels I, II, and III) and MCF-7 (panels IV, V, and VI) cells showing positivity (brown staining) for uPA (II, V) and uPAR (III, VI) antigen. Panels I and IV represent isotype controls.

Table 1. Percentage cell survival at 5 μCi activity of alpha-proteins or at excess concentrations of DTTA-PAI-2 conjugate and PAI-2 compared to controls

Cells	Alpha-PAI-2[a, b, c]		Alpha-BSA[a, c]		DTTA-PAI-2[c] (37.5 μg/ml)	PAI-2[c] (50 μg/ml)
	−	+	−	+		
MDA-MB-231	11 ± 3	40 ± 4	88 ± 5	87 ± 5	98 ± 2	98 ± 1
MCF-7	13 ± 3	40 ± 3	87 ± 5	86 ± 5	98 ± 1	97 ± 2
Leukocytes	98 ± 3	94 ± 6	ND		ND	ND

[a] − and + indicate without and with EGR-CMK pre-treatment. ND = not determined.
[b] Values shown are the means ±SD ($n = 6$, each experiment performed in triplicate).
[c] Values shown are the means ±SD ($n = 2$, each experiment performed in triplicate).
[d] Protein concentration of alpha-PAI-2 was approximately 2.5 μg/ml.

being investigated [25]. We have investigated the use of PAI-2 as the basis for a new therapeutic agent. In this study we describe for the first time the novel compound alpha-PAI-2 and show that it retains reactivity and selectivity towards uPA expressing breast cancer cells *in vitro*.

The ability of PAI-2 to inhibit tumour invasion and metastases in animal models has been demonstrated by several laboratories utilising uPA-overexpressing cancer cells. For example, local invasion of human sarcoma xenografts is limited by stable expression of PAI-2 in the sarcoma cells [42], over-expression of PAI-2 in human melanoma cells inhibits spontaneous metastasis in immuno-compromised mice [43], and pre-treatment of rat mammary cancer cells with recombinant human PAI-2 or slow infusion of the inhibitor with osmotic pumps led to a significant decrease in lung metastasis post intravenous administration [44].

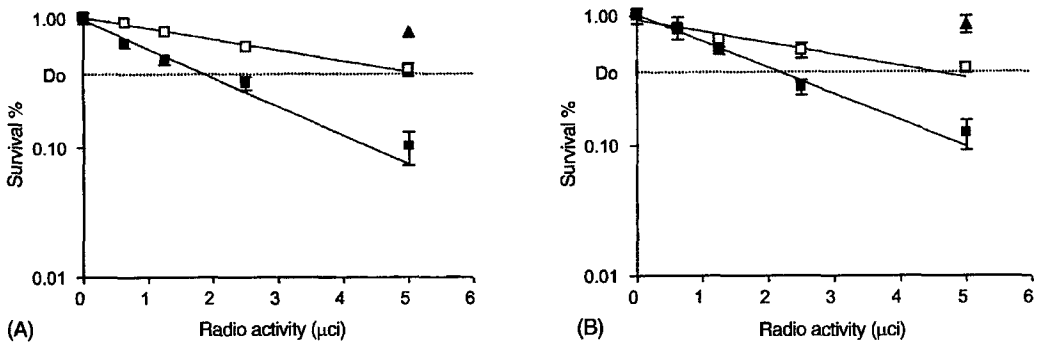

Figure 4. Representative cytotoxicity study of (A) MDA-MB-231 and (B) MCF-7 cells. Cells were either pre-incubated with 0.5 mM of the uPA specific inhibitor EGR-CMK (□) for 15 min at room temperature or with medium alone (■) after plasminogen treatment. Cells were then treated with varying concentrations of alpha-PAI-2 or a single concentration of alpha-BSA (▲), incubated overnight and cell survival measured and expressed as a percentage of cell survival of control cells. Controls consisted of RPMI medium alone. Values shown are the means of two experiments performed in triplicate.

Since ^{125}I-PAI-2 was shown to accumulate in uPA-overexpressing colorectal cancer cell xenografts in mice [36], and PAI-2 levels were shown to be negligible in invasive colorectal cancer tissues that contained high levels of uPA antigen [45], it is likely that exogenous PAI-2 can target invasive tumours in a uPA-dependent manner. Moreover, since localisation studies indicate that quiescent, normal or benign tissues do not contain significant levels of uPA (refer to Introduction) it is unlikely that uPA-targeted therapy will appreciably affect normal tissues.

PAI-1 conjugated to A-chain cholera toxin as the cytotoxic agent or modified PAI-1 conjugated to saporin has been used to target fibrosarcoma cells [46, 47] with moderate cytotoxicity. However, PAI-2 has several distinct advantages over PAI-1 for targeted cancer therapy. Firstly, PAI-2 is 10,000 fold less active than PAI-1 towards tissue type plasminogen activator, the latter having a high affinity for fibrin, indicating that administered PAI-2 would not adversely affect fibrinolysis and hemostasis [35]. PAI-2 is very stable *in vitro* compared to PAI-1 and does not revert to a latent form *in vitro* or *in vivo* compared to PAI-1 [35]. Sustained exposures to PAI-2 are unlikely to cause adverse health effects since the 'abnormally' high PAI-2 levels found during late pregnancy (usually blood levels of PAI-2 are undetectable) are not associated with toxicity [35]. In addition, obstacles associated with targeted immunotherapy, such as large protein size and human anti-mouse antibody responses, both of which require significant antibody manipulation to overcome such problems, are not a concern with PAI-2.

While MDA-MB-231 and MCF-7 express different levels of cell-surface uPA/uPAR, they have similar 37% survival values (i.e., ~2 μCi) when incubated with alpha-PAI-2. This implies that, since only a few alpha particle hits of the nucleus are required to kill a cell [1], compared with thousands for betas, even MCF-7 cells with low uPA levels can still receive a lethal dose at quite low levels of targeted activity. Nevertheless, this is a specific effect at D_0 concentrations of alpha-PAI-2 as pre-treatment of both cell types with the uPA blocking agent EGR-CMK significantly improved cell survival as a result of inhibition of the PAI-2 interaction with cellular uPA. At sub-D_0 concentrations the specific effectiveness of alpha-PAI-2 was more apparent with the uPA/uPAR overexpressing MDA-MB-231 cell line. That alpha-PAI-2 cytotoxicity is significantly mediated via a uPA-dependent mechanism was further confirmed by the lack of cytotoxicity of freshly isolated normal human leukocytes on which cell-surface localised active uPA was not detectable. Furthermore, breast cancer cells incubated with the non- specific alpha-BSA were also minimally affected. Clearly, alpha-PAI-2 is very toxic to targeted cancer cells, whereas non-targeted cells are spared from the radiotoxicity arising from the alpha radiation. These results underscore the potential usefulness of alpha-PAI-2 *in vivo*.

The majority of PAI-2 was derivatised using a 50-fold molar excess of cDTPA and these DTTA-PAI-2 preparations retained their uPA inhibitory activity in full. While the incorporation of ^{213}Bi into DTTA-PAI-2 to form the alpha-PAI-2 construct was found to be efficient and sufficiently stable for *in vitro* studies,

new generation chelates such as cyclohexyl-DTPA may provide better stability *in vivo* [15]. The relative stability of alpha-PAI-2 constructs incorporating cDTPA or cyclohexyl-DTPA will also be considered in future *in vivo* studies.

Conclusions

We have combined the cytotoxicity of an alpha-emitting radioisotope (^{213}Bi) with the targeting potential of PAI-2 towards the uPA system to create a novel construct alpha-PAI-2, a potential new therapeutic for targeted alpha therapy of cancer. The *in vitro* cytotoxicity of alpha-PAI-2 on breast cancer cells was shown to be specific by several means indicating that the cell killing ability of alpha-PAI-2 depends critically on the targeting of cells in a receptor bound, active uPA-dependent manner.

Acknowledgements

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